Hormetic Effects of Carbendazim on the Virulence of *Botrytis cinerea*

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Abstract

The ascomycete plant-pathogenic fungus *Botrytis cinerea* infects more than 1,400 plant species worldwide. Stimulatory effects of sublethal doses of fungicides on plant pathogens are of close relevance to disease management. In the present study, stimulatory effects of carbendazim on the virulence of *B. cinerea* to cucumber plants were investigated. Spraying carbendazim on cucumber plants at 3 to 200 µg/ml had stimulatory effects on the virulence of carbendazim-resistant isolates of *B. cinerea* and the maximum percent stimulations were 16.7 and 13.5% for isolates HBtom451 and HBstr491, respectively. Preconditioned mycelia (i.e., mycelia grown on potato dextrose agar [PDA] amended with carbendazim at concentrations of 10, 50, or 200 µg/ml) also showed increased virulence, and the maximum percent stimulations

for isolates HBtom451 and HBstr491 were 7.9 and 9.5%, respectively. Compared with mycelia grown on PDA without carbendazim, virulence stimulation magnitudes of spraying carbendazim on leaves increased moderately but the concentrations of carbendazim that elicited the maximum stimulation increased 20- and 8-fold for preconditioned isolates HBtom451 and HBstr491, respectively. The time course of infection indicated that virulence stimulation was mediated by a direct stimulation mechanism. Studies of the physiological mechanism for stimulation demonstrated that carbendazim had no significant effects on tolerance to hydrogen peroxide, or on oxalic acid production in *B. cinerea*. These studies will deepen our understanding of quantitative features of hormetic effects of sublethal doses of fungicides on plant pathogens.

The ascomycete fungus *Botrytis cinerea* Pers. is a necrotrophic plant pathogen notorious for its extremely broad host range. *B. cinerea* can infect 586 genera, including more than 1,400 plant species worldwide (Elad et al. 2015; Van Kan et al. 2017). *B. cinerea* mainly infects dicotyledonous plants, many of which are economically important fruit and vegetable crops. Pre- and postharvest losses caused by this pathogen are usually enormous due to its wide host range, huge amounts of conidia, highly resistant sclerotia as a survival structure, and exquisite infection tactics (Amselem et al. 2011; Williamson et al. 2007). No crop cultivar with complete resistance to this pathogen has been reported to date. Due to limited gene resources for plant resistance, fungicides remain a primary approach to controlling *B. cinerea* (Mbengue et al. 2016; Nakajima and Akutsu 2014). Fungicides used for control of *Botrytis* and related genera such as *Sclerotinia* and *Monilinia* account for about 8% of the global fungicide market (Fillinger and Walker 2015). The benzimidazole fungicide carbendazim has been widely employed in China to control a variety of fungal plant pathogens since the 1970s. Extensive and repeated applications of single-site fungicides such as carbendazim have resulted in the emergence and development of fungicide resistance in *B. cinerea* around the world (Konstantinou et al. 2015; Rupp et al. 2017; Saito et al. 2016). Since the earliest report of carbendazim resistance in *B. cinerea* in 1971 (Bollen 1971), different levels of carbendazim resistance have been reported around the world (Liu et al. 2014; Zhang et al. 2006; Zhou and Ye 1987). In spite of extensive and high levels of resistance, carbendazim is still used in China, mainly as a partner ingredient mixed with other fungicides such as pyrimethanil and diethofencarb, to control *B. cinerea* (<http://www.chinapesticide.gov.cn/hysj/index.jhtml>). Therefore, it is of practical significance to investigate stimulatory effects of sublethal doses of carbendazim on *B. cinerea*.

Plant pathogens will be inevitably exposed to sublethal doses of a fungicide in the field due to drift of spray droplets, the decay of the active ingredient, and differential penetration through crop canopies.

Fungicide applied at label-recommended concentrations for functionally sensitive pathogens will become sublethal to the resistant strains (Garzón and Flores 2013). Sublethal doses of fungicide may increase rather than decrease mycelial growth and pathogenicity of plant pathogens. The high-dose inhibitory and low-dose stimulatory effects of fungicide on plant pathogens conform well to a more general biological concept called hormesis (Di et al. 2015; Zhou et al. 2014). A hormesis database has been established and approximately 9,000 hormetic dose-response cases had been included up to 2011 (Calabrese and Blain 2011). Quantitative features and mechanisms of hormesis have been extensively studied and reviewed (Calabrese 2013, 2015a,b, 2016a,b).

With respect to fungicide hormesis, numerous stimulatory responses to sublethal doses of fungicides have been reported such as stimulation of mycelial growth of *Pythium aphanidermatum* by low doses of propamocarb and cyazofamid (Flores and Garzón 2013), mycelial growth and virulence of *P. aphanidermatum* by mfenoxam (Garzón et al. 2011; Moorman and Kim 2004), mycelial growth of *Phytophthora infestans* by metalaxyl (Zhang et al. 1997), spore germination of *Penicillium expansum* by thiabendazole (Baraldi et al. 2003), and mycelial growth of *Lyophyllum palustre* by propamocarb (Landry et al. 2011). Sublethal doses of prothioconazole increase production of the mycotoxin deoxynivalenol in *Fusarium graminearum* (Audenaert et al. 2010). Our laboratory reported stimulatory effects of dimethachlone (Zhou et al. 2014), carbendazim (Di et al. 2015, 2016b), and trifloxystrobin (Di et al. 2016a) on the pathogenicity of *Sclerotinia sclerotiorum*. Although fungicide hormesis has been reported in many plant-pathogenic fungi and oomycetes involving fungicides with different modes of action, to the best of our knowledge, no hormetic responses have been reported for the agriculturally important phytopathogenic fungus *B. cinerea*. In order to design judicious fungicide application strategies for management of *B. cinerea*, it is necessary to explore hormetic effects of fungicides on the virulence of this notorious pathogen. Tolerance to hydrogen peroxide (H₂O₂) may increase the virulence of a pathogen because host plants release reactive oxygen species as an early defense response to initial pathogen challenges (Williams et al. 2011). *B. cinerea* produces oxalic acid both in vitro and in planta. Oxalic acid is an important cofactor in pathogenesis and can stimulate cell-wall-degrading enzymes such as polygalacturonase (Williamson et al. 2007). Investigating effects of sublethal doses of carbendazim on oxalic acid production and tolerance of *B. cinerea* to H₂O₂ may shed light on mechanisms of virulence stimulation. The objectives of the present study were to (i) assess stimulatory effects of sublethal doses of carbendazim on the virulence of *B. cinerea* and (ii) explore potential hormetic mechanisms for virulence stimulation.

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Materials and Methods

Isolates of *B. cinerea*. The two isolates of *B. cinerea* used in the study were kindly provided by Prof. G. Q. Li of Huazhong Agricultural University. Isolate HBtom451 was collected from a diseased tomato plant and isolate HBstr491 was collected from a diseased strawberry plant in Hubei Province. Mycelial colonies of both isolates were maintained on potato dextrose agar (PDA) slants in round bottom polystyrene tubes at 4°C in a refrigerator. The concentrations that reduce mycelial growth by 50% (EC₅₀ values) of carbendazim to the two isolates were higher than 1,000 µg/ml.

Fungicide. Technical-grade carbendazim (98.1% active ingredient; Tian Jin Jin Bei Chemical Co. Ltd.) was dissolved in hydrochloric acid (HCl) at 0.1 mol/liter to produce a 20,000 µg/ml stock solution. The stock solution was stored at 4°C in a refrigerator for less than 2 weeks before being diluted for subsequent experiments.

Stimulatory effects of spraying sublethal doses of carbendazim on the virulence of *B. cinerea* to cucumber plants in pot experiments. The carbendazim stock solution was serially diluted to concentrations of 8,000, 2,000, 500, 100, and 30 µg/ml with HCl at 0.1 mol/liter and the dilute solutions were further diluted 10 times with sterilized water containing 0.1% Triton X-100. Cucumber plants that had grown for 5 weeks in nutrient soils were sprayed with carbendazim dilutions until runoff by a hand-held sprayer (800 ml in volume; Xinmeir Co. Ltd.). Cucumber plants sprayed with HCl at 0.01 mol/liter in 0.1% Triton X-100 were used as the nontreated control. After 1 h of air drying, two plants were inoculated with inverted 2-day-old, 5-mm-diameter mycelial plugs on the adaxial surface of leaves (two leaves per plant, one plug per leaf) for each treatment, including the control. The inoculated cucumber plants were incubated at 25°C for 72 h in a growth chamber with relative humidity maintained at approximately 90%. The diameter of each lesion was measured twice at right angles. The experiment was repeated independently four times.

Stimulatory effects of preconditioning (i.e., growing on carbendazim-amended PDA) on the virulence of *B. cinerea* to detached leaves of cucumber plants. Leaves were detached from cucumber plants that had grown in a growth chamber for 5 weeks. Leaves were inoculated with mycelial plugs cut from preconditioned colonies that had grown for 3 days on PDA media amended with carbendazim at 10, 50, or 200 µg/ml. Mycelial plugs cut from colonies growing on PDA amended with HCl solution at a final concentration of 0.001 mol/liter were used as the nontreated control. The inoculated leaves were incubated at 25°C for 72 h in a growth chamber with relative humidity maintained at approximately 90%. The diameter of each lesion was measured twice at right angles. The experiment was conducted in triplicate and repeated independently five times.

Stimulatory effects of spraying carbendazim on the virulence of preconditioned *B. cinerea*. Mycelia of *B. cinerea* were grown on PDA amended with carbendazim at 200 µg/ml for 2 days as the preconditioning treatment. Five-week-old cucumber plants growing in a growth chamber were used for this experiment. For isolate HBtom451, cucumber plants were sprayed with carbendazim at 10, 30, 100, 200, 400, 800, and 2,000 µg/ml. For isolate HBstr491, cucumber plants were sprayed with carbendazim at 10, 50, 400, and 2,000 µg/ml. Cucumber plants sprayed with HCl at 0.01 mol/liter in 0.1% Triton X-100 were used as the nontreated control. One hour after being treated with carbendazim, leaves were inoculated with inverted 2-day-old mycelial plugs of the preconditioned colonies. The inoculated cucumber plants were incubated as described above. The diameter of each lesion was measured twice at right angles. The experiment was repeated independently three times.

Observations on time course of virulence stimulation. The procedure for recording the time course of virulence stimulation was according to Di et al. (2016a,b), with slight modifications. Leaves detached from 5-week-old cucumber plants were sprayed with carbendazim at 50 µg/ml. Leaves sprayed with HCl at 0.01 mol/liter in 0.1% Triton X-100 were used as the nontreated control. One hour after being treated with carbendazim, leaves were inoculated with mycelial PDA plugs. The inoculated leaves were incubated as described above. At 24, 36, and 48 h postinoculation (HPI), disease symptoms were recorded with a digital camera (Canon Powershot G12).

Observation of the infection process with a scanning electron microscopy (SEM) was according to Di et al. (2015), with minor modifications. Leaves detached from cucumber plants were sprayed with carbendazim at 50 µg/ml and leaves sprayed with HCl at 0.01 mol/liter in 0.1% Triton X-100 were used as the nontreated control. One hour after being treated with carbendazim, three detached leaves from the treatment and three leaves from the control were inoculated with mycelial plugs. At 16 HPI, mycelial plugs were removed and the inoculated sites of leaves were cut into square samples (2 by 2 mm). The samples were immersed in 2.5% (wt/vol) glutaraldehyde solution in sodium phosphate buffer (0.05 M, pH 7.0) at 4°C for 24 h, washed with 0.05 M sodium phosphate buffer, and dehydrated in a graded ethanol series. After being gold coated in a sputter coater (JFC-1600; NTC), the samples were observed for infection cushions with an SEM (JSM-6390/LV; NTC).

Effects of sublethal doses of carbendazim on tolerance of *B. cinerea* to H₂O₂. Inverted fresh mycelial plugs cut from 2-day-old colonies growing on PDA media amended with carbendazim at final concentrations of 2, 10, and 50 µg/ml were inoculated on PDA media amended with H₂O₂ at 0, 20, and 200 µg/ml. Mycelial plugs cut from colonies grown on PDA amended with HCl solution at a final concentration of 0.001 mol/liter were used as the nontreated control. The diameter of each mycelial colony was measured twice at right angles after being incubated at 25°C for 48 h. The experiment was repeated independently three times.

Effects of sublethal doses of carbendazim on oxalic acid production in *B. cinerea*. Mycelia grown on PDA amended with HCl solution at a final concentration of 0.002 mol/liter were used as the nontreated control. After growing at 25°C for 72 h on cellophane-overlaid PDA amended with carbendazim at 50 and 200 µg/ml, mycelia were harvested into a centrifuge tube with a spoon, lyophilized, and ground into fine powder in a mortar. To suspend the powder, 50 mM potassium chloride–hydrogen chloride buffer (2 ml, pH 2.0) was added. After centrifugation at 10,000 rpm for 10 min, the supernatant was used to determine oxalic acid concentrations by a colorimetric method according to Duan et al. (2014) and Di et al. (2015). The assay principle was that content of oxalic acid could be calculated from reductions in absorbance at 510 nm of the complex of iron (III) and sulfosalicylic acid. Absorbance was measured with a UV-1800 spectrophotometer (Shimadzu Corporation). The experiment was repeated independently four times.

Data processing and analysis. Percent stimulations of virulence were calculated by the following formula: Percent stimulation (%) = [(lesion diameter of the treated – lesion diameter of the control)/lesion diameter of the control] × 100%. Curve modeling was performed according to Deng et al. (2001) with the second-order polynomial (quadratic) procedure in the software GraphPad Prism (ver. 5.01; GraphPad Software Inc.). Data of percent stimulation were arcsine square-root transformed for statistical analysis. Two-way analysis of variance with concentration and repeat as two fixed factors was performed with the procedure of Univariate in General Linear Model in the software package SPSS (Statistical Product and Service Solutions, ver. 21.0; SPSS Inc.). The post hoc Dunnett

Fig. 1. Observed values and fitted quadratic curves of virulence stimulations of spraying sublethal doses of carbendazim on *Botrytis cinerea* isolates **A**, HBtom451 and **B**, HBstr491 on potted cucumber plants. Data points represent the mean value of four repeats and error bars denote standard error of the mean.

multiple comparison method was used in SPSS with the nontreated control as the control category. Figures were constructed with the software Microsoft Excel (ver. 2010; Microsoft Corporation) and GraphPad Prism (ver. 5.01).

Results

Stimulatory effects of spraying sublethal doses of carbendazim on the virulence of *B. cinerea* to cucumber plants. Both isolates exhibited significantly increased virulence to cucumber plants sprayed with sublethal doses of carbendazim and the percent stimulations of virulence showed a biphasic inverted U shape (Fig. 1). The R^2 values of the fitted quadratic curves for isolates HBtom451 and HBstr491 were 0.357 and 0.683, respectively. For isolate HBtom451, the stimulation percentage reached a maximum of 16.7% at a dose of 10 $\mu\text{g/ml}$. The carbendazim dose range for statistically significant stimulation was from 10 to 50 $\mu\text{g/ml}$. Carbendazim at 3, 200, and

800 $\mu\text{g/ml}$ had stimulatory effects on the virulence but these stimulations were not statistically significant according to the Dunnett multiple comparison method in the SPSS. For isolate HBstr491, the stimulation percentage reached a maximum of 16.7% at a dose of 50 $\mu\text{g/ml}$, and the carbendazim dose range for statistically significant virulence stimulation was from 3 to 200 $\mu\text{g/ml}$.

Stimulatory effects of preconditioning on the virulence of *B. cinerea* to cucumber plants. With mycelia grown on PDA without fungicide as the nontreated control, preconditioned mycelia of isolate HBtom451 (i.e., growing on PDA media amended with carbendazim at 10 and 50 $\mu\text{g/ml}$) showed statistically significantly higher virulence to detached leaves of cucumber plants (Fig. 2). The maximum percent stimulation was 7.9%. For isolate HBstr491 preconditioned with carbendazim at either 10, 50, or 200 $\mu\text{g/ml}$, significant stimulatory effects on the virulence were recorded, and the maximum percent stimulation was 9.5%.

Stimulatory effects of spraying carbendazim on the virulence of preconditioned *B. cinerea*. Compared with the nontreated control, both isolates preconditioned with carbendazim at 200 $\mu\text{g/ml}$ showed increased virulence to cucumber plants sprayed with carbendazim (Fig. 3). The R^2 values of the fitted quadratic curves for isolates HBtom451 and HBstr491 were 0.572 and 0.807, respectively. For isolate HBtom451, the maximum percent stimulation was 18.0%, at which the concentration of sprayed carbendazim was 200 $\mu\text{g/ml}$. The concentration range for carbendazim to generate statistically significant virulence stimulation was from 10 to 400 $\mu\text{g/ml}$. For isolate HBstr491, the maximum percent stimulation was 27.4%, at which the concentration of sprayed carbendazim was 400 $\mu\text{g/ml}$. The concentration range for carbendazim to generate significant virulence stimulation was from 50 to 400 $\mu\text{g/ml}$.

Time course of virulence stimulation. The earliest symptom could be detected at 24 HPI on leaves treated with carbendazim at 50 $\mu\text{g/ml}$, whereas no necrotic symptom could be discerned by the naked eye for the nontreated control (Fig. 4). From 36 to 48 HPI, disease symptoms developed faster on leaves sprayed with carbendazim, whereas no infection cushions could be detected for the nontreated control. There was more mycelial biomass on leaves treated with carbendazim than on the control (Fig. 5).

Effects of sublethal doses of carbendazim on tolerance of *B. cinerea* to H_2O_2 . H_2O_2 at 20 $\mu\text{g/ml}$ in PDA had no inhibitory effects on mycelial growth rate of *B. cinerea*, whereas H_2O_2 at 200 $\mu\text{g/ml}$

Fig. 2. Stimulatory effects of growing (preconditioning) on carbendazim-amended potato dextrose agar on virulence of *Botrytis cinerea* isolates HBtom451 and HBstr491 on detached leaves of cucumber plants. Bars represent the mean value of five repeats and error bars denote standard error of the mean. Asterisks denote significant difference at an α -level of 0.05 when compared with the nontreated control.

Fig. 3. Observed values and fitted quadratic curves of virulence stimulations of spraying sublethal doses of carbendazim on preconditioned *Botrytis cinerea* isolates **A**, HBtom451 and **B**, HBstr491 on potted cucumber plants. Data points represent the mean value of three repeats and error bars denote standard error of the mean.

Fig. 4. Time course of the infection process of *Botrytis cinerea* isolate HBstr491 on leaves of cucumber plants. Leaves treated with HCl at 0.01 mol/liter in 0.1% Triton X-100 were used as the nontreated control (the first column). The concentration of carbendazim was 50 $\mu\text{g/ml}$. HPI = hours postinoculation.

showed considerable inhibitions against mycelial growth on PDA (Fig. 6). Compared with growing on fungicide-free PDA, growing on PDA amended with carbendazim at 2, 10, or 50 $\mu\text{g/ml}$ did not have statistically significant effects on subsequent mycelial growth rates of either of the two isolates on PDA with or without H_2O_2 . This indicates that carbendazim had no significant effect on tolerance of *B. cinerea* to H_2O_2 .

Effects of sublethal doses of carbendazim on oxalic acid production in *B. cinerea*. For either of the two isolates, growing on PDA amended with carbendazim at 50 or 200 $\mu\text{g/ml}$ did not show any significant effects on oxalic acid production in comparison with the nontreated control (Fig. 7). For each treatment, isolate HBtom451 produced more oxalic acid than isolate HBstr491.

Discussion

The two isolates of *B. cinerea* used in the study were highly resistant to carbendazim, both with EC_{50} values higher than 1,000 $\mu\text{g/ml}$. Thus, sublethal doses of carbendazim to both isolates were relatively high, at a range of 3 to 800 $\mu\text{g/ml}$ for the spraying experiment. The dose range may be lethal to field sensitive *B. cinerea* isolates. In fact, carbendazim is recommended to be sprayed at about 100 to 200 $\mu\text{g/ml}$ in combination with pyrimethanil or diethofencarb for control of gray mold disease caused by *B. cinerea* on grapevines and vegetables (<http://www.chinapesticide.gov.cn/hysj/index.jhtml>). Normal applications of fungicide mixtures with carbendazim as the major component may provide a satisfactory efficacy for sensitive *B. cinerea* but, for carbendazim-resistant isolates, the application doses of carbendazim

may induce virulence stimulation in *B. cinerea*, leading to more severe diseases.

The present study demonstrated that sublethal doses of carbendazim had moderate yet statistically significant stimulatory effects on the virulence of *B. cinerea* to host plant cucumber. Spraying sublethal doses of carbendazim on cucumber plants elicited the maximum virulence stimulations of 16.7 and 13.5% for isolates HBtom451 and HBstr491, respectively. Preconditioned mycelia had the maximum percent virulence stimulations of 7.9 and 9.5% for isolates HBtom451 and HBstr491, respectively. When preconditioned mycelia were inoculated on cucumber plants sprayed with carbendazim, the maximum percent virulence stimulations for isolates HBtom451 and HBstr491 were 18.0 and 27.4%, respectively. These results indicate that stimulatory effects of spraying carbendazim for preconditioned mycelia were higher than those of spraying or preconditioning alone. Double treatments with sublethal doses of carbendazim seemed to have additive or even synergistic effects on virulence stimulation, especially for isolate HBstr491. Furthermore, compared with mycelia grown on carbendazim-free PDA, the concentrations of sprayed carbendazim that elicited the maximum stimulations for preconditioned mycelia increased from 10 and 50 $\mu\text{g/ml}$ to 200 and 400 $\mu\text{g/ml}$ for isolates HBtom451 and HBstr491, respectively. These results have profound implications for field application of fungicides. In the field, some inocula, whether in the form of mycelia or conidia, may survive the latest application of a fungicide. Considerable virulence stimulations may occur when the surviving inocula infect host plants sprayed with sublethal doses of a fungicide.

Temporal aspects of numerous hormetic responses indicate two distinct mechanisms (i.e., direct stimulation mechanism and overcompensation to a disruption of homeostasis). The overcompensation mechanism has been identified in a broad range of biological systems and a wide variety of inducing agents (Calabrese 2001). Although the majority of hormetic responses seem to be caused by an overcompensation mechanism, in some cases, hormetic responses are more likely to be caused by a direct stimulation mechanism. Stimulatory effects of arsenic (Harden and Young 1906), mercuric chloride, and sodium chloride (Branham 1929) on fermentation of yeast seemed to be caused by a direct stimulation mechanism. Our previous studies on the time course of fungicide hormesis demonstrated that stimulatory effects of sublethal doses of carbendazim (Di et al. 2016b), trifloxystrobin (Di et al. 2016a), and flusilazole (Lu et al. 2018) on the virulence of *S. sclerotiorum* were mediated by a direct stimulation mechanism. The present study showed that virulence stimulation of *B. cinerea* by carbendazim was also caused by a direct stimulation mechanism. The direct stimulation mechanism was corroborated by the increased mycelial biomass and earlier formation of infection cushions on cucumber leaves, as indicated by SEM observations on the infection process. The underlying biochemical processes for the direct stimulation mechanism remain to be studied in the future.

H_2O_2 is one of the major reactive oxygen species rapidly released by host plants as early defense responses to an initial pathogen

Fig. 5. Scanning electron micrographs showing the difference in the formation of infection cushions for *Botrytis cinerea* isolate HBtom451 between treated with carbendazim at 50 $\mu\text{g/ml}$ and nontreated control. Samples were collected 16 h after inoculating on detached leaves of cucumber plants.

Fig. 6. Effects of growing on potato dextrose agar amended with carbendazim (Cb) on sensitivity of *Botrytis cinerea* isolates A, HBtom451 and B, HBstr491 to hydrogen peroxide (H_2O_2). Each column represents the mean value of three repeats and error bars denote standard error of the mean.

Fig. 7. Oxalic acid production in *Botrytis cinerea* growing on cellophane-overlaid potato dextrose agar amended with carbendazim. Each column represents the mean value of four repeats. Error bars denote standard error of the mean.

challenge (Williams et al. 2011; Wojtaszek 1997). High tolerance of a pathogenic fungus to H_2O_2 is conducive to its infections. Our earlier studies showed that dimethachlone-treated mycelia of *S. sclerotiorum* exhibited not only increased virulence to host plants but also enhanced tolerance to H_2O_2 (Zhou et al. 2014). However, subsequent studies demonstrated that sublethal doses of carbendazim (Di et al. 2015) and flusilazole (Lu et al. 2018) did not enhance tolerance of *S. sclerotiorum* to H_2O_2 . The present study also showed that sublethal doses of carbendazim did not increase tolerance of *B. cinerea* to H_2O_2 . These results indicate that tolerance to reactive oxygen species is unlikely to be a common mechanism for fungicide hormetic effects on the virulence of fungal plant pathogens.

B. cinerea can secrete oxalic acid in colonized plant tissues (Nakajima and Akutsu 2014). Oxalic acid may enhance activities of pathogenicity factors such as polygalacturonase, aspartic protease, and laccase by lowering ambient pH values (Manteau et al. 2003). Hence, oxalic acid plays an important role in the pathogenesis of *B. cinerea*. The present study showed that virulence stimulation of *B. cinerea* by sublethal doses of carbendazim did not correlate with the secretion of oxalic acid. Previous studies also demonstrated that a stimulatory effect of neither dimethachlone (Zhou et al. 2014) nor carbendazim (Di et al. 2015) on the virulence of *S. sclerotiorum* was caused by oxalic acid. Virulence factors of broad-host-range pathogenic fungi such as *B. cinerea* are numerous (Nakajima and Akutsu 2014) and the interactions between host plants and fungal pathogens are complex (Mbengue et al. 2016). The physiological and biochemical mechanisms for virulence stimulations of sublethal doses of fungicide warrant systematic investigations in the future.

Literature Cited

- Amselem, J., Cuomo, C. A., van Kan, J. A. L., Viaud, M., Benito, E. P., Couloux, A., Coutinho, P. M., de Vries, R. P., Dyer, P. S., Fillinger, S., Fournier, E., Gout, L., Hahn, M., Kohn, L., Lapalu, N., Plummer, K. M., Pradier, J. M., Quévillon, E., Sharon, A., Simon, A., ten Have, A., Tudzynski, B., Tudzynski, P., Wincker, P., Andrew, M., Anthouard, V., Beever, R. E., Beffa, R., Benoit, I., Bouzid, O., Brault, B., Chen, Z., Choquer, M., Collémar, J., Cotton, P., Danchin, E. G., Silva, D. C., Gautier, A., Giraud, C., Giraud, T., Gonzalez, C., Grossetete, S., Güldener, U., Henrissat, B., Howlett, B. J., Kodira, C., Kretschmer, M., Lappartient, A., Leroch, M., Levis, C., Mauceli, E., Neuvéglise, C., Oeser, B., Pearson, M., Poulain, J., Poussereau, N., Quesneville, H., Rascle, C., Schumacher, J., Ségurens, B., Sexton, A., Silva, E., Sirven, C., Soanes, D. M., Talbot, N. J., Templeton, M., Yandava, C., Yarden, O., Zeng, Q., Rollins, J. A., Lebrun, M. H., and Dickman, M. 2011. Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS Genet.* 7:e1002230.
- Audenaert, K., Callewaert, E., Höfte, M., Saeger, S. D., and Haesaert, G. 2010. Hydrogen peroxide induced by the fungicide prothioconazole triggers deoxynivalenol (DON) production by *Fusarium graminearum*. *BMC Microbiol.* 10:112.
- Baraldi, E., Mari, M., Chierici, E., Pondrelli, M., Bertolini, P., and Pratella, G. C. 2003. Studies on thiabendazole resistance of *Penicillium expansum* of pears: Pathogenic fitness and genetic characterization. *Plant Pathol.* 52: 362-370.
- Bollen, G. J. 1971. Acquired resistance to benomyl and some other systemic fungicides in a strain of *Botrytis cinerea* in cyclamen. *Plant Pathol.* 77: 83-90.
- Branham, S. E. 1929. The effects of certain chemical compounds upon the course of gas production by Baker's yeast. *J. Bacteriol.* 18:247-264.
- Calabrese, E. J. 2001. Overcompensation stimulation: A mechanism for hormetic effects. *Crit. Rev. Toxicol.* 31:425-470.
- Calabrese, E. J. 2013. Hormetic mechanisms. *Crit. Rev. Toxicol.* 43:580-606.
- Calabrese, E. J. 2015a. Hormesis: Principles and applications. *Homeopathy* 104: 69-82.
- Calabrese, E. J. 2015b. Hormesis within a mechanistic context. *Homeopathy* 104: 90-96.
- Calabrese, E. J. 2016a. Preconditioning is hormesis part I: Documentation, dose-response features and mechanistic foundations. *Pharmacol. Res.* 110:242-264.
- Calabrese, E. J. 2016b. Preconditioning is hormesis part II: How the conditioning dose mediates protection: Dose optimization within temporal and mechanistic frameworks. *Pharmacol. Res.* 110:265-275.
- Calabrese, E. J., and Blain, R. B. 2011. The hormesis database: The occurrence of hormetic dose responses in the toxicological literature. *Regul. Toxicol. Pharmacol.* 61:73-81.
- Deng, C., Graham, R., and Shukla, R. 2001. Detecting and estimating hormesis using a model-based approach. *Hum. Ecol. Risk Assess.* 7:849-866.
- Di, Y. L., Cong, M. L., Zhang, R., and Zhu, F. X. 2016a. Hormetic effects of trifloxystrobin on aggressiveness of *Sclerotinia sclerotiorum*. *Plant Dis.* 100: 2113-2118.
- Di, Y. L., Lu, X. M., Zhu, Z. Q., and Zhu, F. X. 2016b. Time-course of carbendazim stimulation on pathogenicity of *Sclerotinia sclerotiorum* indicates a direct stimulation mechanism. *Plant Dis.* 100:1454-1459.
- Di, Y. L., Zhu, Z. Q., Lu, X. M., and Zhu, F. X. 2015. Pathogenicity stimulation of *Sclerotinia sclerotiorum* by subtoxic doses of carbendazim. *Plant Dis.* 99: 1342-1346.
- Duan, Y. B., Ge, C. Y., and Zhou, M. G. 2014. Molecular and biochemical characterization of *Sclerotinia sclerotiorum* laboratory mutants resistant to dicarboximide and phenylpyrrole fungicides. *J. Pest Sci.* 87:221-230.
- Elad, Y., Perrot, I., Prado, A. M. C., and Stewart, A. 2015. Plant hosts of *Botrytis* spp. Pages 413-486 in: *Botrytis—The Fungus, the Pathogen and its Management in Agricultural Systems*. S. Fillinger, and Y. Elad, eds. Springer, Berlin.
- Fillinger, S., and Walker, A. S. 2015. Chemical control and resistance management of *Botrytis* diseases. Pages 189-216 in: *Botrytis—The Fungus, the Pathogen and its Management in Agricultural Systems*. S. Fillinger, and Y. Elad, eds. Springer, Berlin.
- Flores, F. J., and Garzón, C. D. 2013. Detection and assessment of chemical hormesis on the radial growth in vitro of oomycetes and fungal plant pathogens. *Dose Response* 11:361-373.
- Garzón, C. D., and Flores, F. J. 2013. Hormesis: Biphasic dose-responses to fungicides in plant pathogens and their potential threat to agriculture. Pages 311-328 in: *Fungicides—Showcases of integrated plant disease management from around the world*. M. Nita, ed. InTech.
- Garzón, C. D., Molineros, J. E., Yanez, J. M., Flores, F. J., Jimenez-Gasco, M. M., and Moorman, G. W. 2011. Sublethal doses of mefenoxam enhance *Pythium* damping-off of geranium. *Plant Dis.* 95:1233-1238.
- Harden, A., and Young, W. J. 1906. The influence of sodium arsenate on the fermentation of glucose by yeast juice. *Proc. Chem. Soc.* 22:283-284.
- Konstantinou, S., Veloukas, T., Leroch, M., Meneses, G., Hahn, M., and Karaoglanidis, G. 2015. Population structure, fungicide resistance profile, and *sdhB* mutation frequency of *Botrytis cinerea* from strawberry and greenhouse-grown tomato in Greece. *Plant Dis.* 99:240-248.
- Landry, J., Martinez, C., and Rochefort, L. 2011. The use of fungicide Nova to mitigate infection of sphagnum by parasitic fungi in the greenhouse. *Botany* 89:655-661.
- Liu, S. M., Gao, X. H., Zhang, Y. H., Li, M., and Mei, X. H. 2014. Detection of the resistance of *Botrytis cinerea* on tomato in Henan Province to three fungicides. *Plant Prot.* 40:144-147.
- Lu, X. M., Zhang, R., Cong, M. L., Li, J. H., and Zhu, F. X. 2018. Stimulatory effects of flusilazole on virulence of *Sclerotinia sclerotiorum*. *Plant Dis.* 102: 197-201.
- Manteau, S., Aboune, S., Lambert, B., and Legendre, L. 2003. Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*. *FEMS Microbiol. Ecol.* 43: 359-366.
- Mbengue, M., Navaud, O., Peyraud, R., Barascud, M., Badet, T., Vincent, R., Barbacci, A., and Raffaele, S. 2016. Emerging trends in molecular interactions between plants and the broad host range fungal pathogens *Botrytis cinerea* and *Sclerotinia sclerotiorum*. *Front. Plant Sci.* 7:422.
- Moorman, G. W., and Kim, S. H. 2004. Species of *Pythium* from greenhouses in Pennsylvania exhibit resistance to propamocarb and mefenoxam. *Plant Dis.* 88: 630-632.
- Nakajima, M., and Akutsu, K. 2014. Virulence factors of *Botrytis cinerea*. *J. Gen. Plant Pathol.* 80:15-23.
- Rupp, S., Weber, R. W. S., Rieger, D., Detzel, P., and Hahn, M. 2017. Spread of *Botrytis cinerea* strains with multiple fungicide resistance in German horticulture. *Front. Microbiol.* 7:2075.

- Saito, S., Michailides, T. J., and Xiao, C. L. 2016. Fungicide resistance profiling in *Botrytis cinerea* populations from blueberry in California and Washington and their impact on control of gray mold. *Plant Dis.* 100:2087-2093.
- Van Kan, J. A., Stassen, J. H., Mosbach, A., Van Der Lee, T. A., Faino, L., Farmer, A. D., Papasotiropoulos, D. G., Zhou, S., Seidl, M. F., Cottam, E., Edel, D., Hahn, M., Schwartz, D. C., Dietrich, R. A., Widdison, S., and Scalliet, G. 2017. A gapless genome sequence of the fungus *Botrytis cinerea*. *Mol. Plant Pathol.* 18:75-89.
- Williams, B., Kabbage, M., Kim, H. J., Britt, R., and Dickman, M. B. 2011. Tipping the balance: *Sclerotinia sclerotiorum* secreted oxalic acid suppresses host defenses by manipulating the host redox environment. *PLoS Pathog* 7:e1002107.
- Williamson, B., Tudzynski, B., Tudzynski, P., and Van Kan, J. A. 2007. *Botrytis cinerea*: The cause of grey mould disease. *Mol. Plant Pathol.* 8:561-580.
- Wojtaszek, P. 1997. Oxidative burst: An early plant response to pathogen infection. *Biochem. J.* 322:681-692.
- Zhang, C. Y., Zhang, Z. X., and Cui, G. R. 2006. Detection and management of the resistance of *Botrytis cinerea* to fungicides in Anhui Province. *Plant Prot.* 32: 32-34.
- Zhang, S., Panaccione, S. G., and Gallegly, M. E. 1997. Metalaxyl stimulation of growth of isolates of *Phytophthora infestans*. *Mycologia* 89:289-292.
- Zhou, F., Liang, H. J., Di, Y. L., You, H., and Zhu, F. X. 2014. Stimulatory effects of sublethal doses of dimethachlon on *Sclerotinia sclerotiorum*. *Plant Dis.* 98: 1364-1370.
- Zhou, M. G., and Ye, Z. Y. 1987. Resistance of plant pathogens to benzimidazole fungicides. *Plant Prot.* 18:31-33.